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(57) Abstract

Described are a process for determining the activity of APP secretase using cellular model systems, those cellular systems being used directly for screening for pharmacological modulators of amyloid βA4 release, and the modulators that can be identified and isolated by that process as pharmaceutical agents for the treatment of Alzheimer's disease. Also described are expression vectors, cells transfected with the expression vectors and the use thereof in the identification of APP secretase modulators, and a kit for determining APP secretase activity.

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Process for the determination of APP secretase modulators and the use thereof as agents in the treatment of Alzheimer's disease

The present invention relates to a process for determining the activity of APP secretase using cellular model systems, those cellular systems being used directly for screening for pharmacological modulators of amyloid βA4 release, and to the modulators that can be identified and isolated by that process as pharmaceutical agents for the treatment of Alzheimer's disease.

The invention relates also to expression vectors, to cells transfected with the expression vectors and to the use thereof in the identification of APP secretase modulators, and to a kit for determining APP secretase activity.

Alzheimer's disease (AD) is the most frequently occurring dementia disease in the elderly. 20% of all people aged 80 suffer from this disease. AD is a neuro-degenerative disease having the following characteristic pathological indicators :

1. Intracellular deposits in the form of neurofibrillary tangels (NFTs), chiefly consisting of the Tau protein.
2. Extracellular amyloid plaques, with the amyloid βA4 peptide as chief component.
3. Amyloid βA4 peptide deposits in the walls of cortical blood vessels.
4. Massive neurone loss in the limbic system and in the cerebral cortex, together with gliosis and atrophy.

Amyloid βA4 peptide, which forms the chief component of extracellular deposits, is a peptide 39-43 amino acids long that is released by two proteolytic activities (β and γ-secretases) from a 695-770 amino acid-long transmembrane-bound amyloid precursor protein (APP). Depending on various cell regulation mechanisms, a third proteolytic activity (α-secretase) can prevent amyloid βA4 release by enzymatic processing within the amyloid βA4 sequence. In addition to the fact that amyloid βA4 is the chief component of extracellular deposits, it has been possible in a large number of *in vitro* and *in vivo* systems to demonstrate the neurotoxic potential of the peptide and accordingly an experimental correspondence between the formation of amyloid βA4 and neurodegeneration.

Decisive findings for clarifying the molecular processes involved in AD come from gene technology. Approximately 3% of all AD cases have an autosomal dominant inheritance, the FAD loci having been mapped on chromosome 21, 19, 14 and 1. Three of the FAD genes have been identified and sequenced so far. It has been

possible to demonstrate that mutations in the amyloid precursor protein (APP on chromosome 21) or in the "presinilin" genes (PS1 on chromosome 14 and PS2 on chromosome 1) segregate with the disease. On the basis of those FAD mutations it has been possible to demonstrate conclusively that amyloid βA4 is causatively involved in the development of the pathological indicators of AD. All mutations found hitherto result in a qualitative or quantitative change in the amyloid βA4 peptide. Modulation (inhibition) of the formation of amyloid βA4 has therefore been one of the main therapeutic approaches.

The simplest modulation of amyloid βA4 formation is effected by a direct or indirect inhibition or stimulation of the proteases involved in amyloid βA4 release (APP secretases α, β or γ). On the basis of various publications (Kojima S. and Omori M. (1992) FEBS 304, 57-60, McDermott J., Biggins J.A. and Gibson A.M. (1992) BBRC 185, 746-752, Nelson R.B., Siman R., Iqbal M.A. and Potter H. (1993) Journal of Neurochemistry 61, 567-577, Sahasrabudhe S.R., Brown A.M., Hulmes J.D., Jacobsen J.S., Vitek M.P., Blume A.J. and Sonnenberg J.L. (1993) Journal of Biological Chemistry 268, 16699-16705, Papastoitsis G., Siman R., Scott R., and Abraham C.R. (1994) Biochemistry 33, 192-199, Matsumoto A. and Fujiwara Y. (1994) Biochemistry 34, 3941-3948, Schönlein C., Löffler J. and Huber G. (1994) BBRC 201, 45-53 and Dreyer R. N., Bausch K. M., Fracasso P., Hammond L. J., Wunderlich D., Wirak D. O., Davis G., Brini C. M., Buckholz T. M., Konig G., Kamarck M. E. and Tamburini P. P. (1994) European Journal of Biochemistry, 224, 265-271) the fact that the APP secretases require a membrane-bound substrate in order to be able to cleave efficiently and specifically must be taken as a starting point.

Hitherto described approaches for identifying protease inhibitors that prevent amyloid βA4 release use synthetic peptide substrates to find corresponding protease inhibitors (Sisodia S.S., Koo E.H., Beyreuther K., Unterbeck A. and Price D.L. (1990) Science 248, 492-495 and LeBlanc A.C. and Gambetti P. (1994) BBRC 204, 1371-1380). The cellular activity and specificity of the inhibitors found in that way is, however, questionable.

It would therefore be desirable to have a process that is suitable for the determination of the activity of APP secretases in intact cells and for the quantitative and differential determination of the APP secretase modulators involved in amyloid βA4 release, and that, in spite of the complexity of the intact cell system, renders possible a quantitative determination of the proteolytic activity being examined, it being possible for the modulators identifiable and obtainable by the process also to be used as pharmaceutical agents in the treatment of Alzheimer's disease.

A process for the determination of the activity of APP secretase in intact cells and for the identification of modulators of APP secretase activity has now been found which is characterised in that

a) an expression vector is prepared that comprises a DNA sequence under the control of a promoter, the DNA sequence coding for a fusion polypeptide comprising
   i) one or more membrane anchor domains,
   ii) one or more recognition sites for an APP secretase and
   iii) one or more polypeptide component(s) releasable by cleavage at the recognition site,
b) the expression vector is introduced into a cell that is capable of expressing one or more APP secretases,
c) the APP secretase activity in the cells obtained according to step (b) is analysed based on the release of the polypeptide component(s) (iii) and
d) optionally the secretase activity is determined in the presence of an added test substance in order to identify modulators that stimulate or inhibit the APP secretase activity.

Suitable promoters include all promoters known in the literature and familiar to the person skilled in the art that are suitable for the purpose in question.
There is to be understood by the polypeptide components mentioned in a) iii), for example, reporter domains.

In particular, a process for the quantitative and differential determination of the APP secretases involved in amyloid βA4 release and for the direct isolation of corresponding modulators is described which is characterised in that

a) first of all a suitable expression vector is prepared by
   i) recombinant fusion of the secretory form of a protein, which secretory form is fused to the recognition sites for APP α- or β-secretase, with
   ii) a suitable transmembrane anchor sequence and
   iii) expression under the control of a suitable promoter,
b) eukaryotic cells are stably transfected with the expression vector so produced,
c) the transfected cells are selected with a suitable marker,
d) the selected stable cells are analysed immunologically or enzymatically by means of the secreted reporter domain using current standard methods and finally
e) with the use of suitable vectors as negative and positive controls, the modulators that stimulate or inhibit the α- or β-secretase activity are determined.

The expression vectors that may be used in process step a) are prepared by

i) recombinant fusion of the secretory form of a protein (reporter), which secretory form is fused to the recognition sites for APP α- or β-secretase, with
ii) a suitable transmembrane anchor sequence and
iii) expression of the protein under the control of a suitable promoter,

The expression vectors that may be used in process step a) may be, preferably, the vectors pCEP/SEAPαsecTM1, pCEP/SEAPαsecTM2, pCEP/SEAPβsecTM1, pCEP/SEAPβsecTM2, pCEP/SEAPβsecswTM1 and pCEP/SEAPβsecswTM2. They may also be functionally equivalent vectors.

The invention relates also to the expression vectors.

The secretory protein that may be used in process step a) may be urokinase (Langer G., Toschi L., Dieckmann J., Schleuning W.-D. (1995) Gene 161, 287-292) or preferably the secretory form of alkaline phosphatase.

The membrane or transmembrane anchor sequence that may be used in process step a) may be the transmembrane domain of any protein or, preferably, the sequences of human APLP2.

The promoter that may be used in process step a) ii) may be any eukaryotic or viral promoter, preferably the Cytomegalovirus promoter.

The cells used in process step b) are eukaryotic cells, preferably human neuroblastoma cells, especially Sy5y cells.

The present invention relates also to cells transfected with the expression vectors, such as eukaryotic cells, preferably human neuroblastoma cells, especially Sy5y cells.

The present invention relates also to the use of the transfected cells for the identification of APP secretase modulators.

The markers used in process step c) may in principle be any customary eukaryotic selection marker known to the person skilled in the art.
The selection marker preferably used in process step c) is hygromycin.

Current standard methods according to process step d) are, for example, Western blots, immunoprecipitation, enzymatic detection reactions etc..

The vectors suitable for use in process step e) are preferably, for the negative control, the vector pCEP/SEAPTM and, for the positive control, the vector pCEP/SEAPsec, or functionally equivalent vectors.

The invention relates also to the modulators obtainable and obtained by the process according to the invention. The modulators stimulate or inhibit the α- and β-secretase activity.
Such modulators are, for example, compounds such as 1,1,2,2-cyclopropane tetracarbonitrile and 9,10-dihydro-12-methylene-ethane anthracen-11-one, which may be used as pharmaceutical compounds in the treatment of Alzheimer's disease.

The invention relates also to pharmaceutical agents or compositions that comprise at least one modulator, optionally together with formulation substances and additives customary in pharmacy.

The present invention relates also to pharmaceutical agents or compositions in the form of tablets, tablets with controlled release of the active ingredient, dragées, pills, capsules, film-coated tablets and film-coated tablets with controlled release of the active ingredient.

The pharmaceutical agents or compositions of the invention are prepared in a manner known *per se* , in a suitable dose, with the customary solid or liquid carriers or diluents and the customary pharmaceutical and commercial excipients according to the desired mode of administration. The preferred preparations are in a form suitable for oral, enteral or parenteral administration. Such forms of administration are, for example, tablets, film-coated tablets, dragées, capsules, pills, powders or depot forms and also suppositories.
Suitable tablets can be obtained, for example, by mixing the active ingredient with known excipients, for example inert diluents such as dextrose, sugars, sorbitol, mannitol, polyvinylpyrrolidone, disintegrators, such as maize starch or alginic acid, binders, such as starch or gelatin, glidants, such as magnesium stearate or talc, and/or agents for obtaining a depot effect, such as carboxypolymethylene, carboxylmethyl cellulose, cellulose acetate phthalate or polyvinyl acetate. The tablets may also consist of several layers.

Correspondingly, dragées can be produced by coating cores, prepared analogously to tablets, with agents conventionally used in dragée coatings, for example polyvinyl-pyrrolidone or shellac, gum arabic, talc, titanium dioxide or sugars. Dragée casings may also consist of several layers, in which the excipients mentioned above for tablets may be used.

Active ingredient-containing capsules may be produced, for example, by mixing the active ingredient with an inert carrier, such as lactose or sorbitol, and encapsulating the mixture in gelatin capsules.

The modulators according to the invention may also be used, in suitable solutions, such as, for example, physiological saline, as infusion or injection solutions.

There are suitable for parenteral administration especially oily solutions, such as, for example, solutions in sesame oil, castor oil and cottonseed oil. Solubilisers, such as, for example, benzyl benzoate or benzyl alcohol, may be added to increase solubility.

It is also possible to incorporate the modulators obtainable and obtained by the process according to the invention into a transdermal system and thus administer them transdermally.

The modulators obtainable and obtained by the process according to the invention may be used in the treatment of Alzheimer's disease.

The present invention relates also to the use of the modulators obtainable by the process in the manufacture of an agent for the treatment of Alzheimer's disease and to the use of the modulators obtained by the process in the manufacture of an agent for the treatment of Alzheimer's disease.

The present invention relates also to a kit for the determination of the activity of APP secretase in intact cells and for the identification of modulators of APP secretase activity, which is characterised in that

a) an expression vector is prepared that comprises a DNA sequence under the control of a promoter, the DNA sequence coding for a fusion polypeptide comprising
   i) one or more membrane anchor domains,
   ii) one or more recognition sites for an APP secretase and
   iii) one or more polypeptide components releasable by cleavage at the recognition site,
b) the expression vector is introduced into a cell that is capable of expressing one or more APP secretases,
c) the APP secretase activity in the cells obtained according to step (b) is analysed based on the release of the polypeptide component(s) (iii) and

d) optionally the secretase activity is determined in the presence of an added test substance in order to identify modulators that stimulate or inhibit the APP secretase activity.

The present invention relates especially to a kit for the quantitative and differential determination of the APP secretases involved in amyloid βA4 release and for the direct isolation of corresponding modulators, which is characterised in that

a) first of all a suitable expression vector is prepared by
   i) recombinant fusion of the secretory form of a protein, which secretory form is fused to the recognition sites for APP α- or β-secretase, with
   ii) a suitable transmembrane anchor sequence and
   iii) expression under the control of a suitable promoter,
b) eukaryotic cells are stably transfected with the expression vector so produced,
c) the transfected cells are selected with a suitable marker,
d) the selected stable cells are analysed immunologically or enzymatically by means of the secreted reporter domain using current standard methods and finally
e) with the use of suitable vectors as negative and positive controls, the modulators that stimulate or inhibit the α- or β-secretase activity are determined.

The eukaryotic cells that may be used in b) are preferably human neuroblastoma cells, especially Sy5y cells.

Description of the drawings

Fig. 1 shows the analysis of the C-terminal fragments after secretase activity. The cells were lysed as described below. The cell extracts were concentrated by precipitation with acetone and separated by SDS-PAGE. The proteins were transferred onto nitrocellulose. Detection was carried out with the antibody APP-CT, which is directed against the C-terminal 20 residues of APP. CT = the C-terminal APP fragment after α-secretase activity.

Fig. 2 shows the analysis of the secreted reporter fragments after secretase activity. The stably transfected cells were labelled for 2 hours with ^{35}S-methionine. The conditioned medium was analysed by means of immunoprecipitation with anti-SEAP (SEAP track) or R1736 (α track) and subsequent SDS-PAGE.

Fig. 3 shows the analysis of the secreted reporter fragments after secretase activity in the presence of ammonium chloride. The stably transfected cells were pre-incubated for 40 minutes, with or without ammonium chloride, in methionine-free medium and then metabolically labelled for 2 hours, with or without ammonium chloride, with ^{35}S-methionine. The conditioned medium was analysed by means of immunoprecipitation with anti-SEAP (SEAP track) and subsequent SDS-PAGE.

Fig. 4 shows stably transfected cells that have been sown in microtitre plates (75 000 per well) and the next day incubated for 90 minutes, with or without ammonium chloride, in cell culture medium. 30 µl of the conditioned medium were used for quantitative analysis with SEAP measurement by chemiluminescence Phospha-Light™, Serva) (A) or SEAP measurement by absorption (B).

To provide a better understanding of the embodiment examples forming the basis of this invention, a list has first of all been drawn up of all the procedures and methods known *per se* necessary for these experiments.

1. Plasmids and vectors used

pCEP (*in vitro* gen), APP cDNA (Kang *et al.*, (1987), Nature 325, 733-736), pCMV/SEAP (Berger *et al.*, (1988), Gene, 66, 1-10), pSP65-PreA4 (Dyrks *et al.*, (1988), EMBO Journal 7, 949-957)

2. Oligonucleotide primers used

The sequences were derived from SEAP (Berger *et al.* (1988), Gene, 66, 1-10), APP (Kang *et al.* (1987), Nature, 325, 733-736) and APLP2 (Wasco *et al.* (1993), Nature Genetics, 5, 95-99).

AP5/868
CGACTCACTA TAGGGAGACC (Seq. ID No. 1)

AP3Cla
CCATCGATCC CCGGGTGCGC GGCGTCGG (Seq. ID No. 2)

bAAP/LP-2
TTCCGACATA GTAGCAGTGC TCTCATTGGC (Seq. ID No. 3)

bALP-2/AP
ACTGCTACTA TGTCGGAATT CTGCATCCAT (Seq. ID No. 4)

bCAP/LP-2
AAATTGGTGC CACTGCGGGA GGACTTCAGT (Seq. ID No. 5)

bCLP-2/AP
CCGCAGTGGC ACCAATTTTT GATGATGAAC (Seq. ID No. 6)

APPClal62
CTCCTTATCG ATAATGGAGA GTTC (Seq. ID No. 7)

KALPClal
CTGAGTATCG ATGCTCTCAT TGGCCTG (Seq. ID No. 8)

FAPLP2XhoI
CCGCTCGAGC TAAATCTGCA TCTGCTCCAG (Seq. ID No. 9)

APPClaI
CGAATCGATT CAGGATATGA AGTTCAT (Seq. ID No. 10)

aAAP/LP-2
GTGGGTTCAA GCAGTGCTCT CATTGGCCTG (Seq. ID No. 11)

aALP-2/AP
AGCACTGCTT GAACCCACAT CTTCTGAAAA (Seq. ID No. 12)

AB LP-2/AP
CTCCCGCAGG AACACCAATT TTTGATGATG (Seq. ID No. 13)

AB AP/LP-2
TTGGTGTTCC TGCGGGAGGA CTTCAGTCTG (Seq. ID No. 14)

TM5
GGACTAGTGG CCCACTGCGG GAGGACTTC (Seq. ID No. 15)

TM3
CCGCTCGAGC CTAAATCTGC ATCTGCTCCA GG (Seq. ID No. 16)

3. Cloning process

Preparation of the plasmid DNA, restriction enzyme digestion, DNA electrophoresis, DNA ligation and bacterial transformation were carried out according to methods generally known to the person skilled in the art (Sambrook *et al.*, (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, N.Y.).

4. RNA preparation

The cells (c. 1 x 10^6) are washed once with PBS buffer and lysed directly in the cell culture dish by the addition of 2 ml of homo-buffer (4M guanidine thiocyanate, 25 mM sodium citrate pH 7; 100 mM β-mercaptoethanol, 0.5% lauroylsarcosine (v/v); 0.1% antifoam A (v/v)). After the lysed cells have been transferred into a 15 ml Sarstedt

tube, the cells are mixed for 10 seconds and 0.2 ml of sodium acetate (2M, pH 4), 2 ml of phenol (pH 4) and 0.4 ml of chloroform are added. The whole is then mixed for a further 10 seconds and centrifuged for 20 minutes (4°C, 10000 g), and the supernatant is transferred into a new Sarstedt tube. The RNA is then precipitated by adding 2 ml of isospropanol and incubating for at least one hour at -20°C. The RNA is subsequently precipitated (20 minutes, 4°C, 10000 g), and the RNA precipitate is dried, dissolved in 0.5 ml of homo-buffer and transferred into a 1.5 ml Eppendorf vessel. After repeated precipitation with isopropanol (0.5 ml isopropanol), the dried RNA is dissolved in 50-100 µl of DEPC/water.

5. RT-PCR

18 µl of commercially available Rtase mix are added to 2 µl of RNA (1 µg). Reverse transcription is then carried out: 10 min. 21°C, then 15 min. 42°C, then 5 min. 99°C and cooling to 4°C. For amplification of the cDNA, 80 µl of commercial PCR mix are added per reaction and the appropriate PCR programme is started: 5 min. 95°C: addition of 0.5 µl of Ampli-Taq-polymerase per reaction after 3.5 min. = "hot start"; followed by 30 cycles with: 1 min. 20 sec. 94°C; 1 min. 20 sec. 55°C; 1 min. 20 sec. 72°C; and a cycle for 10 min. at 72°C, after which the samples are cooled to 4°C.

5.1 PCR

The PCR reactions were carried out as follows:

	recommended conc.	µl
per		**100 µl**
DEPC/water		75
10 times buffer Taq or Pfu		10
dNTP 2.5 mM	200 µM	8
µl primer 1	50 pmol 0.5 µM	2.5
µl primer 2	50 pmol 0.5 µM	2.5
µl template	(0.1-250 ng) 40 ng	1
Taq /Pfu 2.5 U/µl	2.5 U/100 µl	1

Cycles: 25

denaturation	1 min. 95°C
annealing	1 min. 55°C
extension	2 min. 72°C
final extension	5 min. 72°C

6. Cell culture and transfection:

The sub-clone of the neuroblastoma cell line SH-Sy5y was cultured in equal amounts of minimal essential medium (MEM, with Earle's salts and L-glutamine) and Ham's F-12, supplemented with non-essential amino acids (Eagle's formulation), penicillin (50 U/ml), streptomycin (40 µg/ml) and 10 % (v/v) foetal calf serum (FCS).
For stable transfection, 5 times 10^5 cells were sown on 60 mm dishes and incubated overnight at 37°C. Once the cells had achieved approximately 80 % confluence, they were transfected by means of lipofectin. For that purpose the cells were washed three times with Opti-MEM I before the DNA/lipofectin solution was added. The DNA/lipofectin solution was prepared beforehand by mixing diluted DNA (10-20 µg of DNA in 1.5 ml of Opti-MEM) and diluted lipofectin (30-50 µg of lipofectin in 1.5 ml of Opti-MEM) and incubated for 30 minutes at room temperature. After an incubation of 8 hours at 37°C, the cells were cultured in culture medium for a further 24-48 hours and stable lines were selected by the addition of hygromycin B (400 µg/ml the first week, then 300 µg/ml).

7. Metabolic labelling of cell cultures and preparation of cell lysates

After removal of the culture medium, the cells were preincubated for 40 minutes with 2 ml of MEM (6 cm dish) without methionine. The labelling of the cells was carried out with 300 µCi of [^{35}S] methionine in 1.5 ml of MEM without methionine for 2-3 hours. The medium (conditioned medium) was then collected, and the cells were washed with PBS at 4°C and scraped off the dish. For lysis, the centrifuged-off cells were resuspended in 600 µl of sol-buffer (50 mM tris-HCl [pH7.5], 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and commercial protease-inhibitor cocktail) and incubated for 30 minutes on ice. The cell nuclei were centrifuged off at 10000 x g for 5 minutes. The supernatant (cell lysate) was stored at -20°C for further analysis.

8. Immunoprecipitation

The immunoprecipitation was carried out according to the method of Anderson *et al.* (Methods Enzymol. 96, 110-120).

For the immunoprecipitation, the conditioned medium was standardised on 50 mM tris-HCl [pH7.5], 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and commercial protease-inhibitor cocktail. For the preincubation, the conditioned medium and the cell lysate were incubated for 30 minutes with 5 µl of pre-immunoserum and 50 µl (5 mg) of protein A/Sepharose. The samples were briefly centrifuged and the supernatants incubated overnight at 4°C with antibody (10 µl of SEAP, 5 µl of A4CT) and 50 µl (5mg) of protein A/Sepharose. The antibody-protein A complexes were washed three times with washing buffer A (10 mM tris-HCl [pH 7.5], 150 mM NaCl, 0.2 % Triton X-100, 2 mM EDTA), twice with washing buffer B (10 mM tris-HCl [pH 7.5], 500 mM NaCl, 0.2 % Triton X-100, 2 mM EDTA) and once with washing buffer C (10 mM tris-HCl [pH7.5]) and then incubated twice in Laemmli test buffer for 5 minutes at 100°C. The analysis of the labelled proteins was carried out by means of tris-tricine SDS-PAGE and phosphoimaging.

9. Tris-tricine-SDS-polyacrylamide gel electrophoresis

The gel electrophoresis was carried out according to Schägger and Jagow ((1987) Analytical Biochemistry 166, 368-379).

10. Precipitation with acetone

200 µl of the cell lysate are incubated with 1 ml of acetone for 30 minutes at -40°C and then centrifuged off for 10 minutes at 13000 rpm; the supernatant is discarded and the pellet is dried at 37°C. The precipitated protein is dissolved in 40 µl of 8M urea for 15 minutes at 56°C, 40 µl of Laemmli test buffer are added twice and the whole is incubated for a further 15 minutes at 56°C for the purpose of denaturation.

11. Western Blot Analysis

When the gel electrophoresis is complete, the gel is washed for 30 minutes in transfer buffer (25 mM tris, 192 mM glycine, 0.05 % SDS, 20% methanol). The proteins are for 2.5 hours electrophoretically transferred (60 V, 280 mA, 4°C) in transfer buffer onto

0.45 µm of nitrocellulose. After saturation of the nitrocellulose filter in TBST/powdered milk (20 mM tris/pH 7.6, 127 mM NaCl, 0.5% Tween 20, 1% powdered milk) for at least 1 hour at room temperature (RT), the filter is washed twice with TBST (20 mM tris/pH 7.6, 127 mM NaCl, 0.5% Tween 20®) and incubated for a further hour (1-16 hours depending on antibody) at RT with the primary antibody in TBST/powdered milk. After washing three times with TBST, incubation is carried out for at least 30 minutes at RT with secondary antibody (1:7500), coupled to peroxidase, in TBST/powdered milk, followed by washing a further three times with TBST and development with a commercial ECL kit.

12. SEAP Assay:SEAP measurement by chemiluminiscence

All steps are carried out in 96-well microtitre plates. One day before the analysis, 75 000 cells are sown per 96 wells. The analysis is carried out in 200 µl of culture medium plus/minus test substance for from 1 to 20 hours at 37°C. Subsequently, 90 µl of dilution buffer (0.05M tris-HCl, pH 7.4; 0.15M NaCl) are added to 30 µl of the medium and the whole is incubated for 60 minutes at 65°C. After 2 minutes on ice, 100 µl of the heat-treated medium have 100 µl of assay buffer (2M diethanolamine, pH 7.8, 1 mM $MgCl_2$, 20 mM L-homoarginine) added, and the whole is incubated at room temperature for 5 minutes. 100 µl of reaction buffer (0.1M diethanolamine, CSPD) are added to the incubate and, after 20 minutes, the chemiluminescence is measured for 5 seconds in a luminometer.

13. SEAP Assay: SEAP measurement by absorption

All steps are carried out in 96-well microtitre plates. One day before the analysis, 75 000 cells are sown per 96 wells. The analysis is carried out in 200 µl of culture medium with and without test substance for from 1 to 20 hours at 37°C. Subsequently, 100 µl of the medium are incubated at 65°C for 60 minutes. After 2 minutes on ice, 100 µl of the heat-treated medium have 100 µl of assay buffer (2M diethanolamine, pH 7.8, 1 mM $MgCl_2$, 20 mM L-homoarginine) and 20 µl of p-nitrophenol phosphate (120 mM in assay buffer) added. The evaluation is carried out in an ELISA reader at OD 405 as terminal point determination or as kinetics, the amount of enzyme being calculated as follows: 1 mU SEAP = 0.04 A405 increase/min.

The following embodiment Examples illustrate the invention without the invention being limited thereto :

Example 1

Preparation of the cloning and expression vectors

1.1 Vector pBC/SEAP

The N-terminal 511 amino acids of the secretory form of human placental alkaline phosphatase (SEAP; human from the placenta, Berger *et al.* (1988), Gene, 66, 1-10) were amplified by means of PCR from the pCMV/SEAP expression vector (Tropix, Serva, Cat.No. AV10C) as template and the oligonucleotide primers AP5/868 and AP3Cla, and cloned by way of Hind 3 / Cla I into pBluescript SK. The vector pBC/SEAP is obtained.

1.2 Vector SP65/APLP2-CT

The C-terminal 82 amino acids of human APLP2 (Wasco *et al.*, (1993), Nature genetics 5, 95-99) were amplified by means of the above-described RT-PCR and subsequent PCR with the specific oligonucleotide primers TM5 and TM3 and cloned by way of SpeI/XhoI into Sp65/preA4, the vector SP65/APLP2-CT being obtained.

1.3 Vector pCEP/SEAPαsecTM1

The APP fragment , amino acids 604 to 622, referred to here as αsec (numbering according to APP695), was amplified by means of PCR from APP cDNA (Kang *et al.*, (1987), Nature 325, 733-736) as template and the oligonucleotide primers APPClaI and aALP-2/AP. Then, the C-terminal 72 amino acids of APLP2 (Wasco *et al.*, (1993), Nature genetics 5, 95-99), referred to here as TM1, were amplified by means of PCR from vector SP65/APLP2-CT as template and the oligonucleotide primers aAAP/LP-2 and FAPLP2XhoI. The APP fragment and the APLP2 fragment were then fused by means of PCR with the oligonucleotide primers APPClaI and FAPLP2XhoI. The resulting APP/APLP2 hybrid molecule was cloned by way of ClaI / Xho I into pBC/SEAP, vector pBCSEAPαsecTM being obtained. For cellular expression, the

resulting fusion fragment SEAPαsecTM was cloned by way of Hind3/XhoI into the expression vector pCEP4.

1.4 Vector pCEP/SEAPαsecTM2

The APP fragment, amino acids 604 to 615, referred to here as αsec (numbering according to APP695) was amplified by means of PCR from APP cDNA (Kang *et al.*, (1987), Nature, 325, 733-736) as template and the oligonucleotide primers APPClaI and aBLP-2/AP.
Subsequently, the C-terminal 80 amino acids of APLP2 (Wasco *et al.*, (1993), Nature genetics 5, 95-99), referred to here as TM2, were amplified by means of PCR from the vector SP65/APLP2-CT as template and the oligonucleotide primers aBAP/LP-2 and FAPLP2XhoI.
The APP fragment and the APLP2 fragment were then fused with the oligonucleotide primers APPClaI and FAPLP2XhoI by means of PCR. The resulting APP/APLP2 hybrid molecule was cloned by way of ClaI / Xho I into the vector pBC/SEAP resulting in the vector pBCSEAPαsecTM2.
For cellular expression, the resulting fusion fragment SEAPαsecTM was cloned by way of Hind3/XhoI into the expression vector pCEP4.

1.5 Vector pCEP/SEAPβsecTM1

The APP fragment with the amino acids 535 to 604, referred to here as βsec, (numbering according to APP695), was amplified by means of PCR from APP cDNA (Kang *et al.*, (1987), Nature, 325, 733-736) as template and the oligonucleotide primers APPClaI62 and bALP-2/AP. Then, the C-terminal 72 amino acids of APLP2 (Wasco *et al.*, (1993), Nature genetics 5, 95-99), referred to here as TM2, were amplified by means of PCR from the vector SP65/APLP2-CT as template and the oligonucleotide primers bAAP/LP-2 and FAPLP2XhoI. The APP fragment and the APLP2 fragment were then fused with the oligonucleotide primers APPClaI62 and FAPLP2XhoI by means of PCR. The resulting APP/APLP2 hybrid molecule was cloned by way of ClaI / Xho I into pBC/SEAP, resulting in the vector pBCSEAPβsecTM1. For cellular expression, the resulting fusion fragment SEAPβsecTM1 was cloned by way of Hind3/XhoI into the expression vector pCEP4.

1.5a Vector pCEP/SEAPβsecswTM1

Vector pCEP/SEAPβsecswTM1 was generated analogously to pCEP/SEAPβsecTM1 using APP751sw (Urmoneit *et al.* 1995, Journal of Molecular Neuroscience, Vol. 6, 23-32) instead of APP cDNA as template.

1.6 Vector pCEP/SEAPβsecTM2

The APP fragment, amino acids 535 to 614, referred to here as βsec, (numbering according to APP695), was amplified by means of PCR from APP cDNA (Kang *et al.*, (1987), Nature, 325, 733-736) as template and the oligonucleotide primers APPClal62 and bCLP-2/AP. Then, the C-terminal 81 amino acids of APLP2 (Wasco *et al.*, (1993), Nature genetics 5, 95-99), referred to here as TM2, were amplified by means of PCR from the fragment SP65/APLP2-CT as template and the oligonucleotide primers bCAP/LP-2 and FAPLP2Xhol. The APP fragment and the APLP2 fragment were then fused with the oligonucleotide primers APPClal62 and FAPLP2Xhol by means of PCR and the resulting APP/APLP2 hybrid molecule was cloned by way of ClaI / Xho I into pBC/SEAP. The vector pBCSEAPβsecTM2 was obtained. For cellular expression, the resulting fusion fragment SEAPβsecTM2 was cloned by way of Hind3/Xhol into the expression vector pCEP4.

1.6a Vector pCEP/SEAPβsecswTM2

Vector pCEP/SEAPβsecswTM2 was generated analogously to pCEP/SEAPβsecTM2 using APP751sw (Urmoneit *et al.* 1995, Journal of Molecular Neuroscience, Vol. 6, 23-32) instead of APP cDNA as template.

1.7 Vector pCEP/SEAPTM

The C-terminal 70 amino acids of APLP2 (Wasco *et al.*, (1993), Nature genetics 5, 95-99), referred to here as TM, were amplified by means of PCR from SP65/APLP2-CT as template and the oligonucleotide primers KALPClal and FAPLP2Xhol and cloned by way of ClaI / Xho I into pBC/SEAP, resulting in the vector pBCSEAPTM. For cellular expression, the resulting fusion fragment SEAPTM was cloned by way of Hind3/Xhol into the expression vector pCEP4.

1.8 Vector pCEP/SEAPsec

The Hind III / Xba I fragment from the pCMV/SEAP expression vector (Tropix, Serva, Cat. No. AV10C), coding for the secretory form of human placental alkaline phosphatase (SEAP; human from the placenta, Berger *et al.* (1988), Gene, 66, 1-10) was intermediately cloned into pBluescript SK and then cloned by way of Hind3/NotI into the expression vector pCEP4.

Example 2

Transfection of human neuroblastoma cells Sy5y

Sy5y cells are transfected with the above-described expression vectors. On account of the episomal character of the expression vectors, a stable expression is achieved not by dilution and cloning but simply by selection with hygromycin.
Analysis of the stable cells is effected both immunologically (immunoprecipitation with SEAP and APP antibodies) and by means of enzymatic detection of the secreted reporter (SEAP assay).
The stably transfected cells release the reporter (SEAP) into the medium only after α or β-secretase activity. The amount of secreted reporter reflects the level of the respective proteolytic activity. The amount of released reporter can be measured by current standard methods, such as, for example, chemiluminescence, down to sub-nanomolar concentrations. The sensitivity achieved thereby allows a quantitative determination of the α or β-secretase activity in the "high capacity screen"-compatible 96-well format. Published α and β-secretase modulators, such as, for example, phorbol ester and NH_4Cl, exhibit the expected effects in the system described herein. The above-mentioned positive control (pCEP/SEAPsec) and negative control (pCEP/SEAPTM) confer on the system an additional specificity since, as a result, in the course of an HCS falsely positive compounds can easily be identified by secondary screening (inhibition of the secretion caused by a generally toxic influence and inhibition of the alkaline phosphatase is identified by cells transfected with pCEP/SEAPsec; stimulation of the secretion caused by membrane instability is identified by cells transfected with pCEP/SEAPTM).

Example 3

Verification of the processing of the reporter fusion constructs by the corresponding secretase activities

The secretase activities generate, starting from physiological membrane-bound APP, various extracellularly secreted N-terminal fragments (APPsecα, APPsecβ and APPsecγ) and the corresponding intracellular membrane-bound C-terminal fragments (APPsecα ⇒ CT; APPsecβ ⇒ A4CT; APPsecγ ⇒ CT-A4). Provided processing is correct, the expression of the reporter fusion constructs should, after the respective secretase activity, result in a secreted N-terminal fragment (SEAP) and the corresponding intracellular, membrane-bound C-terminal fragment.
Fig. 1 shows a Western blot analysis, developed with an antibody to the C-terminus of APP/APLP2 (anti-APP-CT (Dr. Mönning Schering AG)). Compared with the control CEP (pCEP transfected cells) both the SEAPsecαTM2 and the SEAPsecβTM1 cells exhibit an additionally generated C-terminal fragment which, on account of its size, runs just below the CT fragment of the endogenous APP (CT in the CEP tracks). The corresponding secreted N-terminal fragment can be seen in Fig. 2 after immuno-precipitation with an antibody to SEAP. In addition to the identification of the secreted reporter, Fig. 2 also exhibits proof for the sequence-specific processing of the SEAPsecαTM constructs. The antibody R1736 (Dr. Selkoe/Dr. Haass, Brigham and Woman's Hospital, 221 Long Wood Ave, Boston, MA 02119) recognises the C-terminal amino acid of APP, which is released after α-secretase activity. Immuno-reactivity with R1736 thus indicates sequence-specific α-secretase activity. As can be seen in Fig. 2, track α, only the SEAPsecαTM1 and SEAPsecαTM2 cells secrete significant amounts of SEAP with the R1736 epitope. The small secretion of SEAP with the R1736 epitope in the case of the SEAPsecβTM2 cells results in a residual activity of α-secretase compared with the mutagenised recognition sequence (for details see above under expression vectors). The reporter fusion construct without secretase recognition site (SEAPTM= negative control) shows no reporter activity in the medium.

Example 4

Verification of the regulation of the secretase activities with the fusion constructs as substrate

It is known that β-secretase activity can be inhibited by compounds that render alkaline endosomal/lysosomal compartments (NH_4Cl, chloroquine, methylamine etc.), whilst the α-secretase activity is not affected by those conditions. Fig. 3 shows an immunoprecipitation of the medium with SEAP-antibody after metabolic labelling in the presence or absence of 20 mM NH_4Cl. It can be seen clearly that the processing of SEAPsecβTM2 but not of SEAPsecαTM1 is inhibited under those conditions. In addition to the immunological analysis of the specific regulation of the reporter fusion constructs, the modulation of the reporter secretion was determined quantitatively by means of the SEAP assay.

In contrast to NH_4Cl, phorbol ester (PMA) stimulates α-secretase activity without having an effect on β-secretase activity. It was also possible for that secretase-specific regulation to be reproduced with the reporter fusion constructs. Fig. 4 shows an SEAP assay in which the secreted SEAP was measured as a function of phorbol ester (Fig. 4A) and NH_4Cl (Fig. 4B). The secretion of SEAP in the case of the SEAPαTM cells can be stimulated by PMA by approximately 400%, which agrees very well with the hitherto published data. Under those conditions no, or only a slight stimulation, is observed in the case of the secretion control (SEAPsec cells) and the SEAPsecβTM cells. Also, the specific inhibition of β-secretase activity in the presence of NH_4Cl is reproduced as shown by the SEAP assay in Fig. 4.

Example 5

Overview of the substances tested in the SEAP assay

Using the cellular test system, various compounds were examined for their activity during the processing of the reporter fusion constructs. The substances tested in the SEAP assay were divided into different classes as shown in the following Table.

Substance	Incubation	Effect on the processing of sec-fusion proteins
Acetylcholinesterase inhibitors	2 h, 5 h, 16 h	
Tacrin	10-30 µM	at 30 µM 20-30 % inhibition of the SEAP secretion in all constructs
Galanthamin	10-30 µM	no effect
Physostigmin	10-30 µM	no effect
Growth factors	1-22 h	
PDGF	50 ng/ml	no effect, no action on starved cells
NGF	50 ng/ml	no effect
bFGF	5-50 ng/ml, 4 h	no effect
IGF-1	100 ng/ml, 2 h	no effect
EGF	100 ng/ml, 2 h	no effect
Protease inhibitors	1-2 h	
Pefabloc	200-400 µM	no effect
Aprotinin	0.02-2 µg/ml	no effect
Pepstatin	15 µM, 70* µM	no effect
Leupeptin	10-100 µM	no effect
Phenanthrolin	5-500* µM, 3 h	50 µM inhibits SEAP secretion of all constructs by ~30 %
CGA	0.1-10µM, 2 h, 5 h	no effect
Phosphoramidon	2, 20 µM	no effect
NH_4Cl	10 mM, 2.5 h	inhibits SEAP secretion of SEAPβsec and SEAPβsecsw by ~40 %
BafilomycinA1	0.2 µM	inhibits SEAP secretion of SEAPβsec and SEAPβsecsw by ~35 %
Methylamine	10-30 mM	inhibits SEAP secretion of SEAPβsec and SEAPβsecsw by ~40 %
Cytokine	2 h	
IL-1β	10 ng/ml	no effect on SEAPasec1
IL-4	10 ng/ml	~20 % inhibition of SEAP secretion in all constructs
TNF-α	200 ng/ml	~30 % inhibition of SEAP secretion in all constructs
Con.medium Mikroglia	-/+ LPS stim.	no effect
arachidonic acid	0.3-3 µM	no effect

Substance	Incubation	Effect on the processing of sec-fusion proteins
Inhibitors in the signal transduction	1-3 h	
D609 (PC-PLC-g)	0.2-20* µg/ml	no effect
Wortmannin (PI-3-kinase)	0.1*, 1* µM, 1 h	no effect
Gö6850, Gö6976 (PKC)	1 µM, 1.5 h	~80 % inhibition of the TPA induced SEAP secretion in the case of SEAPα sec1
Activators in the signal transduction	1-3 h	
Carnachol (M-receptor)	1 mM	increases secretion of SEAP in the case of SEAPαsec approx. twofold
TPA (PKC)	0.16, 0.2 µM	increases secretion of SEAP in the case of SEAPαsec approx. two- to four-fold
BMBF305	0.1-3* µg/ml	increases secretion of SEAP in the case of SEAPαsec1
Forskolin (AC)	0.5, 1 µM 3 h preincubation	preincubation with Forskolin inhibits TPA-induced secretion in the case of SEAPasec1 by ~50 %
Oxidative Stress	1-3 h	
Dithiocarbamate (SOD)	0.1-10 µM, 3 h	no effect
H_2O_2	10-100 µM	no effect

For each substance group, the range for the incubation time for all substances of the group is quoted, with incubation times that differ therefrom being noted after the amount. Concentrations marked with * resulted in a visually detectable change in the morphology of the Sy5y cells.

Key :

SEAPαsec: corresponds to SEAPαsecTM1 and SEAPsecαTM2

SEAPαsec1: corresponds to SEAPsecαTM1

SEAPαsec2: corresponds to SEAPsecαTM2

SEAPβsec: corresponds to SEAPβsecTM1 and SEAPβsecTM2

SEAPβsec1 : corresponds to SEAPsecβTM1

SEAPβsec2: corresponds to SEAPsecβTM2

SEAPβsecsw: corresponds to SEAPβsecswTM1 and SEAPβsecswTM2

SEAPβsecsw1 : corresponds to SEAPsecβswTM1

SEAPβsecsw2: corresponds to SEAPsecβswTM2

Example 6

Detection of pharmacological modulators using the process according to the invention and the plasmids and cells according to the invention

The process according to the invention is used as described above for screening for pharmacological modulators of amyloid βA4 release.
The following compounds could be found as modulators
1,1,2,2-cyclopropane tetracarbonitrile (1) and
9,10-dihydro-12-methylene-ethane anthracen-11-one (2)
Compound (1) and its preparation are described by Wideqvist (Ark. Kemi, 20 B No. 4, p 6, 1945) and Hart *et al.* (J. Org. Chem. 31, 2784-2789, 1966).
Compound (2) and its preparation is described by E. Snyder (J. Org. Chem. 25, 1328-1330, 1960), Hart *et al.* (J. Amer. Chem. Soc., 95, 6294, 1973) and Varecht *et al.* (J. Chem. Res. Miniprint, 1979).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
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(ii) TITLE OF INVENTION: Process for the determination of APP secretase modulators and the use thereof as agents in the treatment of Alzheimer's disease

(iii) NUMBER OF SEQUENCES: 16

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: DE 19641180.7
(B) FILING DATE: 24-SEP-1996

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CGACTCACTA TAGGGAGACC

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CCATCGATCC CCGGGTGCGC GGCGTCGG

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TTCCGACATA GTAGCAGTGC TCTCATTGGC

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

ACTGCTACTA TGTCGGAATT CTGCATCCAT

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AAATTGGTGC CACTGCGGGA GGACTTCAGT

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CCGCAGTGGC ACCAATTTTT GATGATGAAC

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CTCCTTATCG ATAATGGAGA GTTC

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CTGAGTATCG ATGCTCTCAT TGGCCTG

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CCGCTCGAGC TAAATCTGCA TCTGCTCCAG

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CGAATCGATT CAGGATATGA AGTTCAT

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GTGGGTTCAA GCAGTGCTCT CATTGGCCTG

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

AGCACTGCTT GAACCCACAT CTTCTGAAAA

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CTCCCGCAGG AACACCAATT TTTGATGATG

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

TTGGTGTTCC TGCGGGAGGA CTTCAGTCTG

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GGACTAGTGG CCCACTGCGG GAGGACTTC

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CCGCTCGAGC CTAAATCTGC ATCTGCTCCA GG

Patent Claims

1. Process for the determination of the activity of APP secretase in intact cells and for the identification of modulators of APP secretase activity, characterised in that
   a) an expression vector is prepared that comprises a DNA sequence under the control of a promoter, the DNA sequence coding for a fusion polypeptide comprising
      i) one or more membrane anchor domains,
      ii) one or more recognition sites for an APP secretase and
      iii) one or more polypeptide components releasable by cleavage at the recognition site,
   b) the expression vector is introduced into a cell that is capable of expressing one or more APP secretases,
   c) the APP secretase activity in the cells obtained according to step (b) is analysed based on the release of the polypeptide component(s) (iii) and
   d) optionally the secretase activity is determined in the presence of an added test substance in order to identify modulators that stimulate or inhibit the APP secretase activity.

2. Process for the quantitative and differential determination of the APP secretases involved in amyloid βA4 release and for the direct isolation of corresponding modulators, characterised in that
   a) first of all a suitable expression vector is prepared by
      i) recombinant fusion of the secretory form of a protein, which secretory form is fused to the recognition sites for APP α- or β-secretase, with
      ii) a suitable transmembrane anchor sequence and
      iii) expression under the control of a suitable promoter,
   b) eukaryotic cells are stably transfected with the expression vector so produced,
   c) the transfected cells are selected with a suitable marker,
   d) the selected stable cells are analysed immunologically or enzymatically by means of the secreted reporter domain using current standard methods and finally
   e) with the use of suitable vectors as negative and positive controls, the modulators that stimulate or inhibit the α- or β-secretase activity are determined.

3. Process according to claim 2, characterised in that, in accordance with step b) human neuroblastoma cells are stably transfected with the expression vector so produced.

4. Process according to claim 1 or 2, characterised in that the expression vectors that may be used in process step a) are the vectors pCEP/SEAPαsecTM1, pCEP/SEAPαsecTM2, pCEP/SEAPβsecTM1, pCEP/SEAPβsecTM2, pCEP/SEAPβsecswTM1 and pCEP/SEAPβsecswTM2.

5. Process according to any one of claims 2 to 4, characterised in that the secretory protein that may be used in process step a) i) is the secretory form of alkaline phosphatase or urokinase.

6. Process according to any one of claims 2 to 5 characterised in that the transmembrane anchor sequences that may be used in process step a) ii) are the sequences of human APLP2.

7. Process according to any one of claims 2 to 6, characterised in that the promoter that may be used in process step a) iii) renders possible expression in eukaryotic systems.

8. Process according to any one of claims 2 to 7, characterised in that the promoter that may be used in process step a) iii) is a eukaryotic or viral promoter.

9. Process according to any one of claims 2 to 8, characterised in that the promoter that may be used in process step a) iii) is the Cytomegalovirus promoter.

10. Process according to any one of claims 1 to 9, characterised in that the cells used in process step b) are Sy5y cells.

11. Process according to any one of claims 2 to 10, characterised in that the selection marker used in process step c) is hygromycin.

12. Process according to any one of claims 2 to 11, characterised in that the vectors suitable for use in process step e) are, for the negative control, the vector pCEP/SEAPTM and, for the positive control, the vector pCEP/SEAPsec.

13. Modulator, obtainable in accordance with the process according to any one of claims 1 to 12.

14. Modulator, obtained in accordance with the process according to any one of claims 1 to 12.

15. Modulator according to claim 13 or 14, characterised in that it is the compound 1,1,2,2-cyclopropane tetracarbonitrile or 9,10-dihydro-12-methylene-ethane anthracen-11-one.

16. Pharmaceutical agent, comprising at least one modulator according to any one of claims 13 to 15 with customary formulation substances and additives.

17. Pharmaceutical agent according to claim 16, for the treatment of Alzheimer's disease.

18. Use of a modulator obtainable by the process according to any one of claims 1 to 15 in the manufacture of an agent for the treatment of Alzheimer's disease.

19. Use of a modulator obtained by the process according to any one of claims 1 to 15 in the manufacture of an agent for the treatment of Alzheimer's disease.

20. Expression vector prepared by
   i) recombinant fusion of the secretory form of a protein (reporter), which secretory form is fused to the recognition sites for APP α- or β-secretase, with
   ii) a suitable transmembrane anchor sequence and
   iii) expression of the protein under the control of a suitable promoter.

21. Expression vector according to claim 20, characterised in that the vector is pCEP/SEAPαsecTM1, pCEP/SEAPαsecTM2, pCEP/SEAPβsecTM1, pCEP/SEAPβsecTM2, pCEP/SEAPβsecswTM1 or pCEP/SEAPβsecswTM2.

22. Cell, transfected with an expression vector according to claim 20 or 21.

23. Human neuroblastoma cell, transfected with an expression vector according to claim 20 or 21.

24. Sy5y cell, transfected with an expression vector according to claim 20 or 21.

25. Use of the transfected cells according to any one of claims 22 to 24 in the identification of APP secretase modulators.

26. Kit for the determation of the activity of APP secretase in intact cells and for the identification of modulators of APP secretase activity, characterised in that

a) an expression vector is prepared that comprises a DNA sequence under the control of a promoter, the DNA sequence coding for a fusion polypeptide comprising

i) one or more membrane anchor domains,

ii) one or more recognition sites for an APP secretase and

iii) one or more polypeptide components releasable by cleavage at the recognition site,

b) the expression vector is introduced into a cell that is capable of expressing one or more APP secretases,

c) the APP secretase activity in the cells obtained according to step (b) is analysed based on the release of the polypeptide component(s) (iii) and

d) optionally the secretase activity is determined in the presence of an added test substance in order to identify modulators that stimulate or inhibit the APP secretase activity.

27. Kit, according to claim 26, for the quantitative and differential determination of the APP secretases involved in amyloid βA4 release and for the direct isolation of corresponding modulators, characterised in that

a) first of all a suitable expression vector is prepared by

i) recombinant fusion of the secretory form of a protein, which secretory form is fused to the recognition sites for APP α- or β-secretase, with

ii) a suitable transmembrane anchor sequence and

iii) expression under the control of a suitable promoter,

b) eukaryotic cells are stably transfected with the expression vector so produced,

c) the transfected cells are selected with a suitable marker,

d) the selected stable cells are analysed immunologically or enzymatically by means of the secreted reporter domain using current standard methods and finally

e) with the use of suitable vectors as negative and positive controls, the modulators that stimulate or inhibit the α- or β-secretase activity are determined.

28. Kit, according to claim 27, characterised in that in b) human neuroblastoma cells are stably transfected with the expression vector so produced.

29. Kit, according to claim 27, characterised in that in b) Sy5y cells are stably transfected.

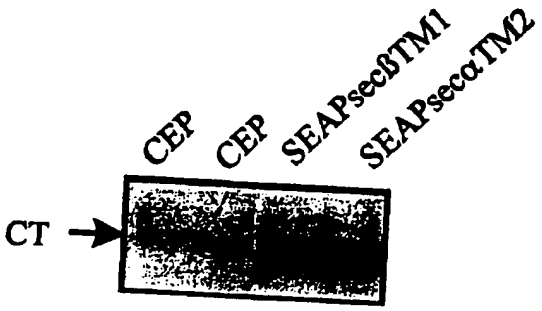
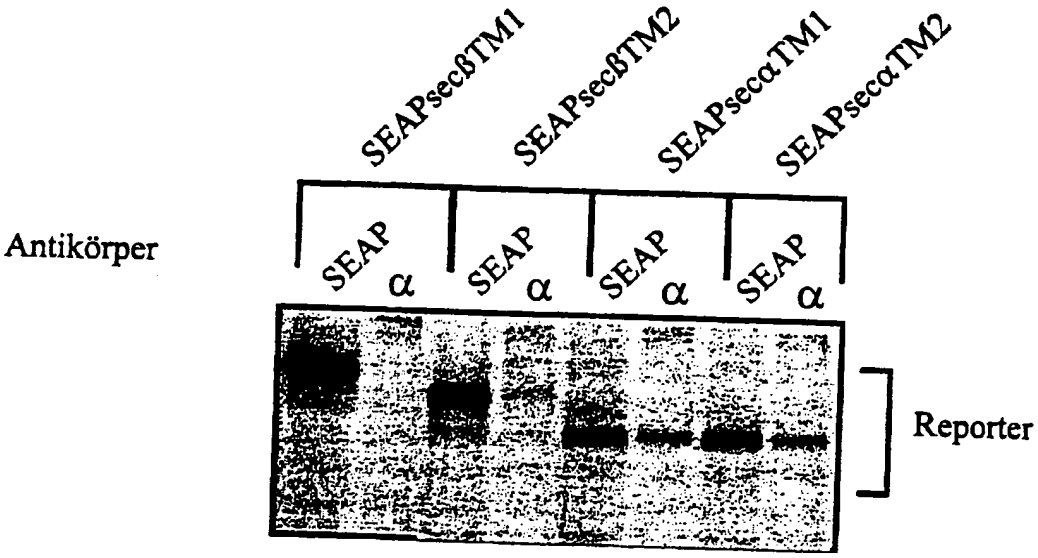


Fig. 1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SEAPsecαTM2
Antikörper
SEAP
α
SEAP
α
SEAP
α
SEAP
α
Reporter

Fig. 2

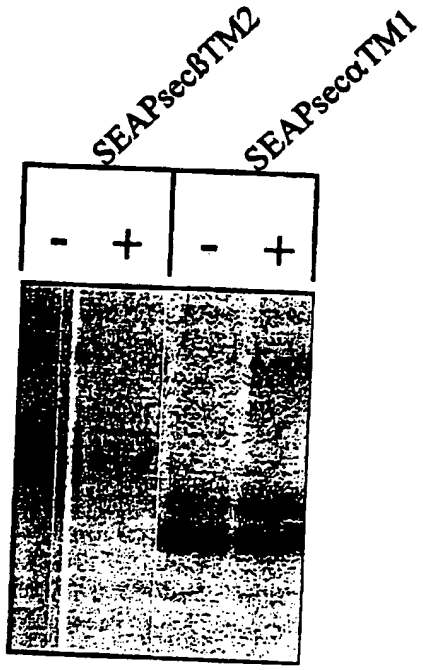
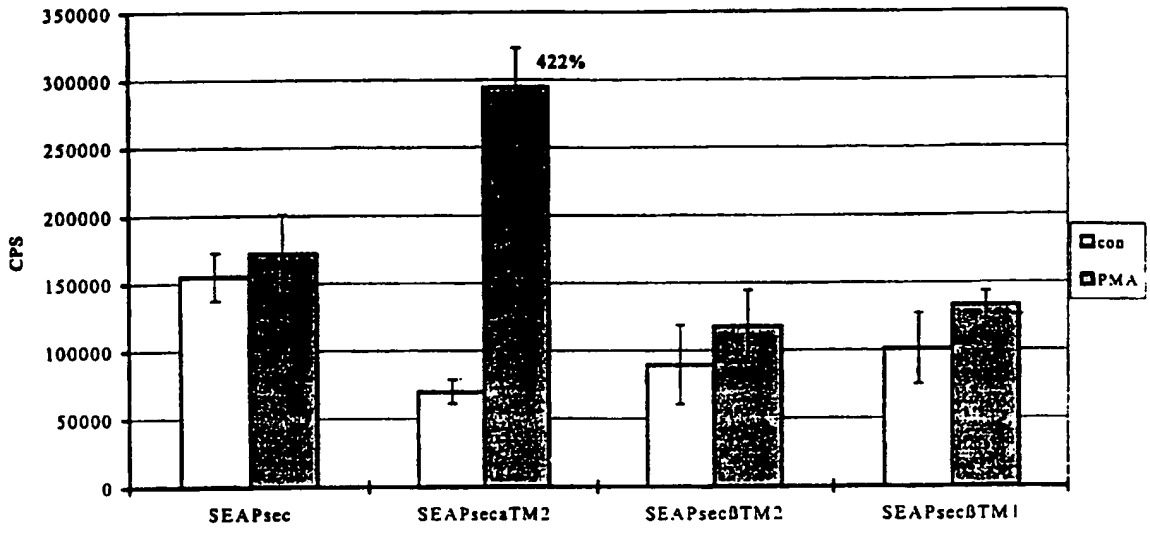


Fig. 3

A)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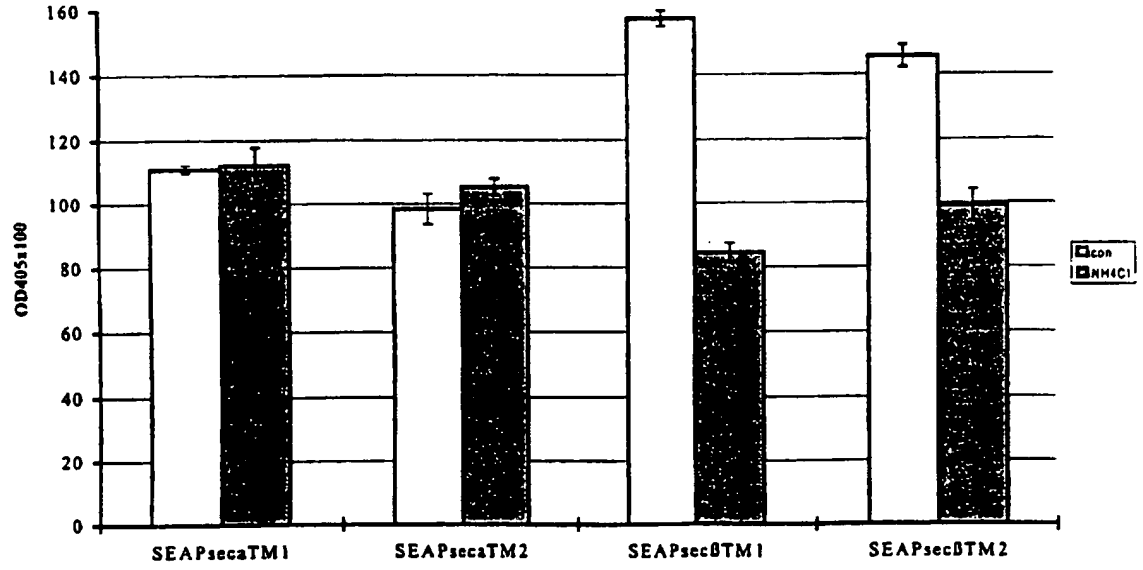
SEAPsecßTM2
SEAPsecßTM1
con
PMA

B)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120
100
80
60
40
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SEAPsecaTM1
SEAPsecaTM2
SEAPsecßTM1
SEAPsecßTM2
con
NH4Cl

Fig. 4

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(71) Applicant *(for all designated States except US)*: SCHERING AKTIENGESELLSCHAFT [DE/DE]; Patente, Müllerstrasse 178, Postfach 65 03 11, D–13342 Berlin (DE).

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(54) Title: PROCESS FOR THE DETERMINATION OF APP SECRETASE MODULATORS AND THE USE THEREOF AS AGENTS IN THE TREATMENT OF ALZHEIMER'S DISEASE

(57) Abstract

Described are a process for determining the activity of APP secretase using cellular model systems, those cellular systems being used directly for screening for pharmacological modulators of amyloid βA4 release, and the modulators that can be identified and isolated by that process as pharmaceutical agents for the treatment of Alzheimer's disease. Also described are expression vectors, cells transfected with the expression vectors and the use thereof in the identification of APP secretase modulators, and a kit for determining APP secretase activity.

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INTERNATIONAL SEARCH REPORT

Inter. nal Application No
PCT/EP 97/05149

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C12N15/62 C12N15/55 C12N15/85 C12N9/16
C12N9/64 C12N5/10 C12Q1/42 A61K31/12 A61K31/275

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EFTHIMIOPOULOS ET AL.: "STUDY OF PHORBOL ESTER EFFECT ON ALZHEIMER AMYLOID PRECURSOR PROCESSING:SEQUENCE REQUIREMENTS AND INVOLVEMENT OF A CHOLERA TOXIN SENSITIVE PROTEIN" JOURNAL OF NEUROSCIENCE RESEARCH, vol. 38, 1994, pages 81-90, XP002057045 see the whole document ---	1-14, 20-29
X	URMONEIT B ET AL: "INHIBITION OF BETAA4 PRODUCTION BY SPECIFIC MODULATION OF BETA-SECRETASE ACTIVITY" JOURNAL OF MOLECULAR NEUROSCIENCE, vol. 6, no. 1, 1 January 1995, pages 23-32, XP000577185 see the whole document ---	1-14, 16-29

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
16 June 1998	22.07.98

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CITRON M ET AL: "GENERATION OF AMYLOID BETA PROTEIN FROM ITS PRECURSOR IS SEQUENCE SPECIFIC" NEURON, vol. 14, no. 3, 1 March 1995, pages 661-670, XP000577165 see the whole document	1-12, 20-29
A	EP 0 569 777 A (MILES INC) 18 November 1993 see the whole document	1-12, 20-29
X		13,14, 16-19
X	US 5 523 314 A (BUE-VALLESKEY ET AL.) 4 June 1996 see abstract see column 31, line 47 - column 36, line 42; claims	13,14, 16-19
A	A.M. BROWN ET AL.: "EVALUATION OF CATHEPSINS D AND G AND EC 3.4.24.15 AS CANDIDATE BETA-SECRETASE PROTEASES USING PEPTIDE AND AMYLOID PRECURSOR PROTEIN SUBSTRATES" JOURNAL OF NEUROCHEMISTRY, June 1996, pages 2436-2445, XP000602767 see abstract see page 2444, left-hand column, last paragraph	13,14, 16-19
A	WO 94 10569 A (SCHENK DALE B ;SCHLOSSMACHER MICHAEL G (AT); SELKOE DENNIS J (US);) 11 May 1994 see the whole document	1-12, 20-29
A	HAASS C ET AL: "THE SWEDISH MUTATION CAUSES EARLY-ONSET ALZHEIMER'S DISEASE BY BETA-SECRETASE CLEAVAGE WITHIN THE SECRETORY PATHWAY" NATURE MEDICINE, vol. 1, no. 12, December 1995, pages 1291-1296, XP000602042 see the whole document	1-12, 20-29
A	EP 0 586 790 A (GEN HOSPITAL CORP ;MASSACHUSETTS INST TECHNOLOGY (US)) 16 March 1994 see the whole document	1-12, 20-29

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WASCO ET AL.: "ISOLATION AND CHARACTERIZATION OF APLP2 ENCODING A HOMOLOGUE OF THE ALZHEIMER'S ASSOCIATED AMYLOID BETA PROTEIN PRECURSOR" NATURE GENETICS, vol. 5, 1993, pages 95-99, XP002057046 cited in the application see the whole document	1-12, 20-29
A	EP 0 327 960 A (HOFFMANN LA ROCHE) 16 August 1989 see the whole document	5
P,X	US 5 652 092 A (VITEK MICHAEL PETER ET AL) 29 July 1997 see the whole document	1-12, 20-29
P,X	WO 96 40885 A (ATHENA NEUROSCIENCES) 19 December 1996 see abstract see page 25, line 21 - page 28, line 2; claims	13,14, 16-19
X	I.A. MCDONALD ET AL.: "INHIBITION OF AMYLOID BETA-PROTEIN FORMATION FOR THE TREATMENT OF ALZHEIMER'S DISEASE" ABSTRACTS OF PAPERS AMERICAN CHEMICAL SOCIETY, vol. 212, no. 1-2, August 1996, page 212 XP002068227 see abstract	13,14, 16-19
X	WO 96 12486 A (SMITHKLINE BEECHAM) 2 May 1996 see the whole document	13,14, 16-19
X	J.T. LEMLEY ET AL.: "THE CRYSTAL AND MOLECULAR STRUCTURE OF 1,1,2,2-TETRACYANOCYCLOPROPANE" ACTA CRYSTALLOGR. SECT. B, vol. B32, no. 1, 1976, pages 35-40, XP002068228 see the whole document	13-15
X	E.I. SNYDER ET AL.: "SOME SYNTHESES AND STRUCTURES IN THE 9,10-DIHYDRO-9,10-ETHANOANTHRACENE SERIES" J. ORG. CHEM., vol. 25, 1960, pages 1328-1330, XP002068229 cited in the application see the whole document	13-15

-/--

3

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	H. HART ET AL.: "METHYLENEKETENES BY IRRADIATION OF alpha,beta-UNSATURATED KETONES" J. AM. CHEM. SOC., vol. 95, 1973, pages 6294-6301, XP002068230 cited in the application see the whole document -----	13-15

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 97/05149

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

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Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Subject 1 Claims 1-12,20-29
Subject 2 Claims 13-19

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 97/05149

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0569777 A	18-11-1993	AU 679675 B	10-07-1997
		AU 3711093 A	18-11-1993
		CA 2095888 A	12-11-1993
		JP 8268911 A	15-10-1996
		MX 9302725 A	31-08-1994
		NZ 247575 A	25-09-1996
		NZ 272218 A	27-07-1997
		WO 9513084 A	18-05-1995
		ZA 9303249 A	11-03-1994
		AU 5726494 A	04-07-1994
		CA 2151927 A	23-06-1994
		EP 0694076 A	31-01-1996
		JP 8508464 T	10-09-1996
		WO 9413319 A	23-06-1994
US 5523314 A	04-06-1996	US 5716975 A	10-02-1998
		AU 676843 B	27-03-1997
		AU 4621893 A	17-03-1994
		CA 2105598 A	11-03-1994
		CN 1091006 A	24-08-1994
		CZ 9301814 A	16-03-1994
		EP 0587377 A	16-03-1994
		FI 933946 A	11-03-1994
		HU 70184 A	28-09-1995
		IL 106877 A	10-03-1998
		JP 6192091 A	12-07-1994
		MX 9305444 A	31-05-1994
		NO 933198 A	11-03-1994
		NZ 248573 A	27-02-1996
		PL 300335 A	21-03-1994
		US 5661168 A	26-08-1997
		ZA 9306492 A	02-03-1995
WO 9410569 A	11-05-1994	AU 687747 B	05-03-1998
		AU 4844493 A	24-05-1994
		CA 2105903 A	27-04-1994
		EP 0667959 A	23-08-1995
		JP 8502587 T	19-03-1996
		US 5593846 A	14-01-1997

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Intern ial Application No
PCT/EP 97/05149

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0586790 A	16-03-1994	CA 2093735 A	21-10-1993
		JP 6128294 A	10-05-1994
EP 0327960 A	16-08-1989	JP 2005862 A	10-01-1990
US 5652092 A	29-07-1997	US 5656477 A	12-08-1997
		US 5693478 A	02-12-1997
		US 5703209 A	30-12-1997
		AU 3835893 A	04-11-1993
		CA 2095421 A	02-11-1993
		EP 0584452 A	02-03-1994
		JP 7132094 A	23-05-1995
		NZ 247499 A	26-04-1996
		ZA 9303084 A	24-11-1993
WO 9640885 A	19-12-1996	US 5744346 A	28-04-1998
		AU 6383396 A	30-12-1996
WO 9612486 A	02-05-1996	AU 3843195 A	15-05-1996
		BG 101500 A	30-01-1998
		BR 9509433 A	16-09-1997
		CZ 9701251 A	16-07-1997
		EP 0786998 A	06-08-1997
		HU 77007 A	02-03-1998
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